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Contrasting Patterns of Diterpene Acid Induction by Red Pine and White Spruce to Simulated Bark Beetle Attack, and Interspecific Differences in Sensitivity Among Fungal Associates

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Abstract Conifers possess a suite of physiochemical defenses that protect their subcortical tissues from bark beetle - fungal complexes. These defenses include rapid induction of terpenoids and phenolics at the site of attack. Studies of the distribution, induction, and bioactivity of conifer terpenoids have focused heavily on monoterpenes. We assessed induction of diterpene acids in white spruce (*Picea glauca*) and red pine (*Pinus resinosa*) to fungal associates of two bark beetles, and the responses of four spruce beetle (*Dendroctonus rufipennis*)—associated fungi to three diterpene acids. Constitutive phloem contents differed between species, in that red pine had extremely low concentrations of diterpene acids, whereas white spruce had substantial constitutive levels. Induction differed quantitatively. Both red pine and white spruce exhibited marked increases, but red pine underwent greater increases and achieved higher concentrations than white spruce. Induction also differed qualitatively in that red pine showed lower diversity and fewer compositional changes during induction than white spruce. In red pine,fungal inoculation accompanying wounding elicited greater increases than wounding alone, but in white spruce total concentrations were higher following wounding alone. Spruce beetle fungal symbiont growth varied among species and compounds. Some diterpenes elicited both stimulatory and inhibitory effects on fungi, depending on concentration. All four fungi exhibited higher tolerances compared to those associated with pine bark beetles in previous studies. Variation in tolerances to, and potentially metabolism of, diterpene acids by symbionts may reflect differences in constitutive levels between spruce and pine, and partially explain differences in concentrations achieved during induction.

Keywords Plant defense · Terpenoids · *Ophiostoma* · *Leptographium* · *Ips* · *Dendroctonus*

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Introduction

Conifers possess sophisticated, multicomponent defenses that can protect trees from attack by insects, pathogens, and insect-microbial complexes. Several species can sometimes overcome these defenses, particularly subcortically feeding bark beetles and associated microbes (Carroll et al. 2006; Smith et al. 2012). Trees resist bark beetle attacks through integrated physical and chemical defenses, which include autonecrosis, biosynthesis of secondary chemicals, and resins that flow from storage sites in ducts and glands to the point of attack (Franceschi et al. 2005; Kane and And Kolb 2010; Lewinsohn et al. 1993; Lieutier et al. 1992; Rosner and Hannrup 2004). These highly viscous resins can physically delay, expel or entomb beetles, are rich in compounds that can repel host-searching adults, intoxicate adults and their brood, inhibit

growth and germination of microbial associates, and interfere with the pheromone signaling that beetles need to overcome tree defenses through synchronous attacks (Erbilgin et al. 2006; Everaerts et al. 1988; Raffa et al. 2005).

The effects of compounds in conifer resin and phloem often are dose-dependent, both in the magnitude and direction of their impacts on beetles and associated microbes. For example, low concentrations often benefit beetles by stimulating entry, synergizing the attractiveness of their pheromones, or stimulating pheromone synthesis. High concentrations of the same chemicals, however, typically deter entry, reduce attraction to pheromones, and are lethal (Bohlmann 2012; Erbilgin et al. 2006; Seybold et al. 2006; Wallin and Raffa 2000). The flow of resin from a wound, and the composition and concentrations of its chemical constituents, are highly inducible, particularly in response to biotic agents (Clark et al. 2014).

Conifers contain a diversity of secondary chemicals, with the predominant compounds being monoterpenes, diterpene acids, and phenolics (Franceschi et al. 2005; Kersten et al. 2006; Raffa et al. 2005). Of these, monoterpenes and phenolics have received the most attention. Monoterpenes are highly inducible, and undergo rapid biosynthesis within just a few days (Huber et al. 2004; Zhao et al. 2011; Zulak and Bohlmann 2010). Monoterpene proportions and concentrations can greatly alter in response to stimuli, with the extents varying among conifer species (Raffa et al. 2005). Monoterpenes can exert behavioral aversive, physiologically sublethal, and toxic effects on bark beetles (Manning and Reid 2013; Raffa and Smalley 1995; Wallin and Raffa 2000), and inhibit the beetles' symbiotic fungi (Klepzig et al. 1996). They also may be exploited by bark beetles as host recognition cues (Elkinton et al. 1981), and as components in pheromone biosynthesis (Blomquist et al. 2010).

Phenolics, likewise, are present in constitutive phloem tissue and undergo induction upon attack, although to a lesser extent than monoterpenes (Hammerbacher et al. 2011; Klepzig et al. 1996). Phenolics contribute to tree defense (Faccoli and And Schlyter 2007), and may function alongside monoterpenes in a complementary manner, with monoterpenes typically having greater activity against the beetles, and phenolics typically having greater activity against the beetles' fungal symbionts (Klepzig et al. 1996).

Conifer diterpene acids have received less attention in terms of their concentrations, inducibilities, sources of variation, and bioactivities (Erbilgin et al. 2006; Villari et al. 2012; Zhao et al. 2010). Diterpene acids are synthesized from isopentenyl diphosphate produced via the mevalonate-independent pathway in plastids (Keeling and Bohlmann 2006; Trapp and Croteau 2001). Biosynthesis is regulated by several terpene synthases whose transcriptions can be elicited by biotic stimuli (Hall et al. 2013; Hamberger et al. 2011). Several studies have shown that diterpene acids can deter larval hymenopteran and lepidopteran folivore feeding and development (Larsson et al. 2000; Powell and Raffa 1999; Schmelz et al. 2011; Wagner et al. 1983). Their effects on subcortical insects, however, are less clear. Sitka and white spruce resistance to white pine weevil has been correlated with higher diterpene acid concentrations (Tomlin et al. 1996). In contrast, diterpene acids exert minor effects on the pine engraver, *Ips pini* (Say). Instead, they greatly reduced growth and germination of *I. pini's* fungal symbiont *Ophiostoma ips* (Rumbold), exhibiting higher activity than any monoterpene or phenolic previously tested (Kopper et al. 2005). Furthermore, growth of the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) fungal associates *Ophiostoma montium* (Rumbold) and *Grosmannia clavigera* (Robinson-Jeffrey & R.W. Davidson) are greatly reduced by diterpene acids (Boone et al. 2013). These fungal associates are critical to beetle success by helping to overcome tree defenses (Kim et al. 2008; Lieutier et al. 2009) and contributing to beetle nutrition (Bleiker and Six 2007).

Red pine (*Pinus resinosa* Aiton) is distributed throughout much of the Great Lakes region of North America. The major insect attacking mature trees is *I. pini*. White spruce (*Picea glauca* Moench) is widely distributed across North America. Its major tree killing insect is the spruce beetle, *Dendroctonus rufipennis*. The predominant symbiotic fungus of *I. pini* is *O. ips* (Klepzig et al. 1991). *Dendroctonus rufipennis* (Kirby) is associated with several symbiotic fungi, of which *Leptographium abietinum* (Peck) is most prominent (Aukema et al. 2005; Bentz and Six 2006; Haberkern et al. 2002). The constitutive and induced monoterpene and phenolic compositions of red pine and white spruce have been reported in several previous studies, (Klepzig et al. 1996; Raffa and Smalley 1995; Werner and Illman 1994). Diterpene acid contents of white spruce in Wisconsin were reported by Kersten et al. (2006), but there is no information on whether, and if so how, they are induced by attack. Likewise, we have no information on how fungi associated with *D. rufipennis* respond to diterpene acids. We assessed induction of diterpene acids in red pine and white spruce, and responses of *D. rufipennis* -associated fungi to three diterpene acids to complement prior work on sensitivity of *O. ips*.

Methods

Site Selection and Defense Induction Ten mature red pine and white spruce trees were selected from two planted stands in Dane County, WI, USA. Experiments were conducted in late June 2004, as previous work demonstrates this is within the period of strong induction of defense chemicals to simulated beetle attack (Raffa and Smalley 1988). Constitutive phloem samples were removed from bark and placed in screw-cap vials, transported to the laboratory on ice, and stored at −20 °C until processing. Induction treatments were

administered at the same time on the same trees. There were two induction treatments, mechanical wounding and mechanical wounding accompanied by fungal application. These methods are described in detail in Raffa and Smalley (1995) and Boone et al. (2011). Treatments were administered by removing a 2-cm plug of phloem and bark, and applying fungal inoculum with a metal syringe. The phloem and bark plug then was replaced, and adjacent tissues rapidly seal the wound. The fungus used for red pine inoculation was *O. ips*, isolated from a red pine tree recently killed by *I. pini* (Kopper et al. 2005), and the fungus used for white spruce inoculation was *L. abietinum* isolated from *D. rufipennis* (Reynolds 1992). After 10 days, the cork bark over the mechanical or biotic inoculation points was removed, the necrotic lesions were excised from phloem with a scalpel, and the samples were placed into screw-top vials, transported to the laboratory on ice, and stored at −20 °C until processing as with the constitutive controls.

Diterpene Acid Analysis Diterpene acids were analyzed by combined high performance liquid chromatography (HPLC)—spectrophotometry, using the method of Kersten et al. (2006). Constitutive and induced phloem samples were removed from the freezer and chopped with a razor blade into approximately 2-mm square pieces and extracted in 4 ml methanol. Extracts were filtered through glass wool and returned to storage at −20 °C. Prior to HLPC analysis, extracts were filtered through 0.45-μm syringe filters.

After preparation, (20 μl) samples were injected onto a Hewlett-Packard (Palo Alto, CA, USA) series 1050 HPLC fitted with an Alltech (Deerfield, IL, USA) Alltima C18 column (5 μm, 250×4.6 mm). Separation was achieved with an isocratic mobile phase with a ternary solvent system (85, 5, and 10 %; methanol, 5 % acetic acid, water, respectively) with a flow rate of 1 ml/min. Diterpene acid analytical standards were purchased from Helix Biotech (British Columbia, Canada). Abietic, neoabietic, palustric, levopimaric, and dehydroabietic acids, with purities>90 % were prepared in 95 % ethanol. One unknown compound previously was found to have a similar spectrum as abietic acid, but a different retention time (Kersten et al. 2006). We refer to this compound as x_abietic acid. HPLC data were analyzed with Agilent ChemStation (Santa Clara, CA, USA) for LC 3D at 240, 268, 282, and 300 nm, allowing selective analyses of abietanes as reported in Kersten et al. (2006). Standard stock solutions were quantified spectrophotometrically at λ_{max} values: abietic at 241 nm (24,150 M^{-1} cm^{-1}), neoabietic at 252 nm (24, 540 M^{-1} cm^{-1}), palustric at 266 nm (9060 M^{-1} cm^{-1}), levopimaric at 272 nm (5800 M^{-1} cm^{-1}), and dehydroabietic at 268 nm (698 M^{-1} cm^{-1}) and 276 nm (774 M^{-1} cm^{-1}).

Fungal Bioassays Bioassays were conducted to test the effects of diterpene acids on four species of fungi associated with D. rufipennis. Fungal associates included *L. abietinum, Pesotum sp.*, *Ophiostoma piceaperdum* (Rumbold), and *Ophiostoma bicolor* (Rumbold). Reynolds (1992) initially isolated *L. abietinum*, and other strains were isolated by Haberkern et al. (2002). Abietic acid, dehydroabietic acid, and isopimaric acid were dissolved separately in acetone to enable additions to growth medium. Diterpene acids or acetone were amended to 2 % malt extract agar (20 g malt extract; 15 g agar in one liter of water) at concentrations 0.1, 0.5, and 1 % (w / v) for abietic and dehydroabietic acid, and 0.1 and 0.5 % for isopimaric acid according to Kopper et al. (2005). Diterpene acids were added to medium in 50 μl acetone and allowed to evaporate until acetone odor dissipated (~6 h) prior to bioassay. Diterpene acids appeared to be evenly distributed in the media. The fungal growth assay as previously described (Klepzig et al. 1996; Kopper et al. 2005). Briefly, active fungal culture growing on malt extract agar was added to the center of a 7-ml scintillation vial containing growth media. Bioassays were maintained for 3 days in dark conditions at 24 °C after which linear growth (diameter) of the culture was calculated. Each treatment contained 10 replicate vials.

Statistical Analyses Statistical analyses were performed using the R (v. 3.0.1) statistical programming environment (R Core Team 2013). Diterpene acid concentrations and composition were log{y +1} transformed to help meet normality assumptions. Each tree was included as a random effect. Generalized linear models (GLMs) were fit using treatment as a fixed effect using the package lme4 with the 'lme' function (Bates et al. 2014). An ANOVA was conducted on the model, and pairwise comparisons were performed using a Tukey HSD corrected P-value with the 'ghlt' function in the package multcomp (Hothorn et al. 2014). For red pine diterpene acids, constitutive samples were excluded from composition analyses due to detectable levels only occurring in one tree (see Results). Therefore, comparisons of proportionate compositions of individual compounds in red pine between mechanical wounding and fungal inoculation were conducted using a paired *t*-test. Because diterpene acids were present in quantifiable amounts in constitutive as well as induced spruce phloem, comparisons of proportionate composition were made among all three treatments. Diterpene acid compositions were analyzed using GLMs followed by an ANOVA.

Statistical analyses of fungal bioassay results were performed by ANOVA, using fungal growth as a response variable, and diterpene acid type and concentration as factors. Pairwise comparisons within a fungal species between chemical treatments were conducted using the least significant difference method using the function 'LSD.test' in the package agricolae (de Mendiburu 2014).

Results

Red Pine and White Spruce Undergo Rapidly Induced Local Changes in Diterpene Acid Contents in Response to Simulated Bark Beetle Attack Diterpene acids were detected in both red pine and white spruce phloem tissues (Fig. 1). Abietic, neoabietic, palustric, levopimaric, and dehydroabietic acids were detected in both red pine and white spruce. X_abietic acid was detected in white spruce, but not in red pine. In constitutive phloem samples, the average total diterpene acid concentrations were substantially higher in white spruce than red pine. Diterpene acids in non-induced phloem were at detectable levels only in a single red pine tree out of the ten that were assessed. In contrast, all of the above diterpene acids were detected in the constitutive phloem from all ten white spruce trees. In trees that received mechanical wounding only, mean total diterpene acid quantities were not very different in phloem of these two tree species, being 52± 12.5 μg / mg in red pine and 50±5.0 μg / mg in white spruce. However, red pine exposed to fungal challenges had substantially greater (5.3 ×) concentrations of diterpene acids than did white spruce.

Red pine diterpene acids underwent substantial increases in concentrations following induction treatments (Fig. 1a). The largest increase in concentration was in levopimaric acid ($F_{2, 18}$=126.4; P<0.001), and the smallest was in dehydroabietic acid ($F_{2, 18}$=66.81; P<0.001). In red pine, all diterpene acids had higher concentrations following the fungal inoculation treatment than with the mechanical wound. This difference corresponded to a 1.67 to 1.85-fold increase. Levopimaric acid constituted the majority of the composition of diterpene acids in red pine (~40 %), while dehydroabietic acid comprised the least (~6 %). Palustric, abietic, and neoabietic acids comprised the remainder of the composition in close to equal percentages (16–19 %). With one exception, there were no significant differences in diterpene composition between wounded and fungal challenged treatments (Fig. 1c). Only levopimaric acid was found in significantly higher composition in fungal inoculations than in mechanically wounded treatments ($t_{df=9}$=3.01; P=0.015). However, this difference was not substantial, and hence probably not biologically significant (40 % compared to 43 % of total composition).

Diterpene acids of white spruce also underwent concentration increases in induction treatments (Fig. 1b). However, response to the attack stimulus differed from that of red pine in being accompanied by marked compositional changes (Fig. 1d). Diterpene acids in white spruce significantly increased in abundance when challenged with a mechanical

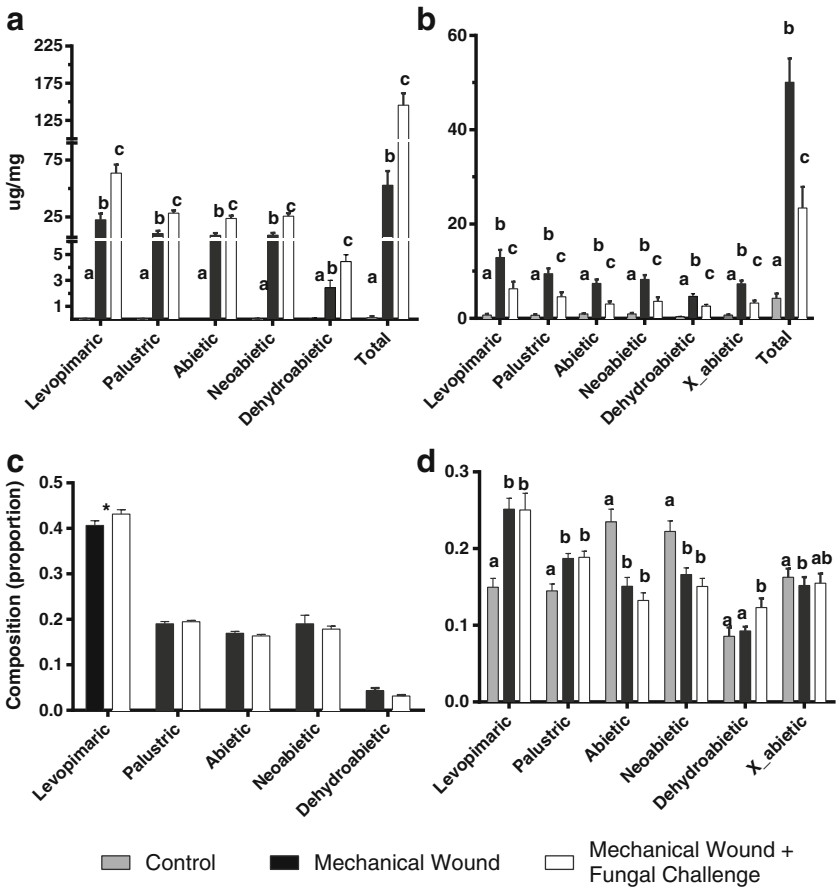

Fig. 1 Concentration (**a** and **b**) and composition (**c** and **d**) of diterpene acids in *red pine* (**a** and **c**) and *white spruce* (**b** and **d**). Treatments included an unwounded control, mechanical wounding, and mechanical wounding with inoculation beetle-associated fungi. Red pine was inoculated with *Ophiostoma ips* and *white spruce* was inoculated with *Leptographium abietinum*. With *red pine*, composition of control trees was not included in the analysis due to undetectable levels of diterpene acids in nine out of the ten trees sampled. *Different letters* (Panels *a*, *b*, *d*; ANOVA) and asterisks (Panel *c*; *t*-test) represent statistically significant differences using log(y+1) transformed data (Tukey HSD corrected; α=0.05)

wound. However, unlike red pine, the mechanical wounding treatment elicited the greatest increases in diterpene acid concentrations. When fungi were administered, there was a 44–60 % lower accumulation of diterpene acids. The response of white spruce also differed from that of red pine in that the relative composition of individual diterpene acids in white spruce differed among control and induction treatments (Fig. 1d). Compositions of levopimaric acid ($F_{2,\ 18}$=28.48; P<0.001) and palustric acid ($F_{2,\ 18}$=17.28; P<0.001) were the most abundant in the induction treatments, where they increased relative to the controls by 67 and 30 %, respectively. The most abundant diterpene acids in the controls, abietic acid ($F_{2,\ 18}$=41.84; P<0.001) and neoabietic acid ($F_{2,\ 18}$=24.27; P<0.001), decreased in induction treatments by 35 and 25 %. Diterpene acid composition of the phloem collected from fungal inoculations were not significantly different from responses to mechanical wounding, with the exception of dehydroabietic acid ($F_{2,\ 18}$=6.86; P=0.006).

Fungi Associated with D. rufipennis Vary in Response to Diterpene Acids Fungal associates of *D. rufipennis* exhibited differential growth responses among fungal species and among chemical treatments (Table 1). There was a significant effect of fungal species ($F_{3,\ 324}$=795.6; P<0.001), diterpene acid in growth medium ($F_{2,\ 18}$=69.09; P<0.001), and the interaction between the two ($F_{2,\ 18}$=32.20; P<0.001) on mycelial growth. In the controls, *L. abietinum* and *Pesotum sp.* grew the least over the course of the experiment, *O. piceaperdum* grew a moderate amount, and *O. bicolor* had the greatest growth. Compared to acetone controls, media amended with 1.0 % abietic acid inhibited the growth of *L. abietinum*, *Pesotum sp*., and *O. piceaperdum*, but stimulated the growth of *O. bicolor*. Different responses were observed in the media amended with dehydroabietic acid and isopimaric acid. Growth of *L. abietinum* was initially stimulated under low concentrations (0.10 %) of dehydroabietic acid, but was inhibited at 1.0 %. *Ophiostoma piceaperdum* exhibited a non-linear response to dehydroabietic acid; its growth was more greatly inhibited by low concentrations than at 1.0 %. *Pesotum sp*., and *O. bicolor* growth also was stimulated compared to controls by the inclusion of dehydroabietic acid. Isopimaric acid stimulated the growth of *L. abietinum*, *Pesotum sp*., and *O. bicolor*, but this stimulatory effect of isopimaric acid on *Pesotum sp*. growth was reduced at 0.5 %. Unlike the other fungi, *O. piceaperdum* growth was unaffected by isopimaric acid.

Table 1 Growth (mm) of fungi associated with spruce beetle on media containing commercially available diterpene acids (w / v). Numbers are means (± std. err.) Different letters represent statistically significant differences within a fungal isolate (P<0.05)

	Acetone control	Abietic acid			Dehydroabietic acid			Isopimaric acid		*F*-value	*P*
		0.10 %	0.50 %	1.00 %	0.10 %	0.50 %	1.00 %	0.10 %	0.50 %	df=8, 81	
Leptographium abietinum	6.90±0.26 cd	7.45±0.33 c	6.10±0.12 e	5.80±0.08 e	8.20±0.17 b	6.35±0.11 de	6.00±0.18 e	8.85±0.20 a	8.30±0.41 ab	24.41	< 0.01
Pesotum sp.	6.60±0.31 c	5.30±0.17 d	5.75±0.3 d	5.10±0.1 d	9.90±0.22 b	9.85±0.2 b	10.15±0.28 b	11.30±0.39 b	10.20±0.4 a	80.12	< 0.01
Ophiostoma piceaperdum	8.45±0.26 bc	8.85±0.17 ab	8.00±0.3 c	7.10±0.18 d	7.10±0.19 d	5.55±0.14 e	8.10±0.21 c	8.50±0.27 b	9.10±0.23 abc	24.87	< 0.01
Ophiostoma biccolor	11.25±0.23 e	11.75±0.23 cde	12.15±0.21 bc	11.85±0.26 cd	12.5±0.13 a	11.6±0.12 de	11.7±0.23 cde	12.45±0.14 ab	12.85±0.13 a	7.06	< 0.01

Discussion

Both red pine and white spruce undergo dramatic, rapid changes in the diterpene acid concentrations of their phloem tissues in response to mechanical wounding and or fungal

inoculation. These results complement previous findings regarding changes in monoterpene (Raffa and Smalley 1988; Werner and Illman 1994; Zhao et al. 2010) and phenolic (Brignolas et al. 1998; Villari et al. 2012) components of conifer responses to native bark beetle-associated fungi. However, the magnitude of diterpene acid induction was substantially higher compared to these other chemical groups. Diterpene acids of red pine increased by over 1000 fold, compared to monoterpenes at 32–40 fold, and phenolics at approximately 2.5 fold (Klepzig et al. 1995; Raffa and Smalley 1995). Constitutive concentrations, and hence the role of induction, varied markedly between these conifer species. To our knowledge, there are no known cases in which previously absent biologically active conifer terpenes or phenolics have been shown to appear in response to a bark beetle - vectored fungus. In red pine, induction of diterpene acids constituted an apparent phytoalexin response, in that these compounds were undetectable in the constitutive phloem of almost all trees studied. In contrast, diterpene acids were present in low constitutive levels of each white spruce. A second difference is that diterpene acids rose to much higher concentrations in red pine than in white spruce following both mechanical and fungal treatments. A third difference is that red pine accumulated higher levels of diterpene acids in response to combined fungal-mechanical than mechanical-alone wounding, but the opposite occurred in white spruce. A fourth difference is that compositional differences in the relative proportions of individual diterpene acids occurred in response to fungal inoculation vs. mechanical wounding in white spruce but not red pine. Fifth, the composition of white spruce diterpene acids was somewhat more diverse than that of red pine. This parallels previous work comparing the monoterpene fractions in *Pinus* and *Abies*, in which compositional changes were induced to a lesser degree in *Pinus* (Raffa et al. 2005).

Previous work indicates that diterpene acids have strong antifungal properties (Boone et al. 2013; Kopper et al. 2005; Kusumoto et al. 2010). However, the effects of diterpene acids on fungal associates of *D. rufipennis* appear to be generally lower and more complex than for fungal associates of other bark beetles, which are summarized in Fig. 2. The *I. pini* fungal symbiont, *O. ips*, was inhibited by all three compounds tested, and the *D. ponderosae* symbionts, *G. clavigera* and *O. minus*, were both inhibited by the single compound tested. However, responses of *D. rufipennis* symbionts were more varied. Abietic acid inhibited the growth of *L. abietinum*, *Pesotum sp.*, and *O. piceaperdum*, but not *O. bicolor*.

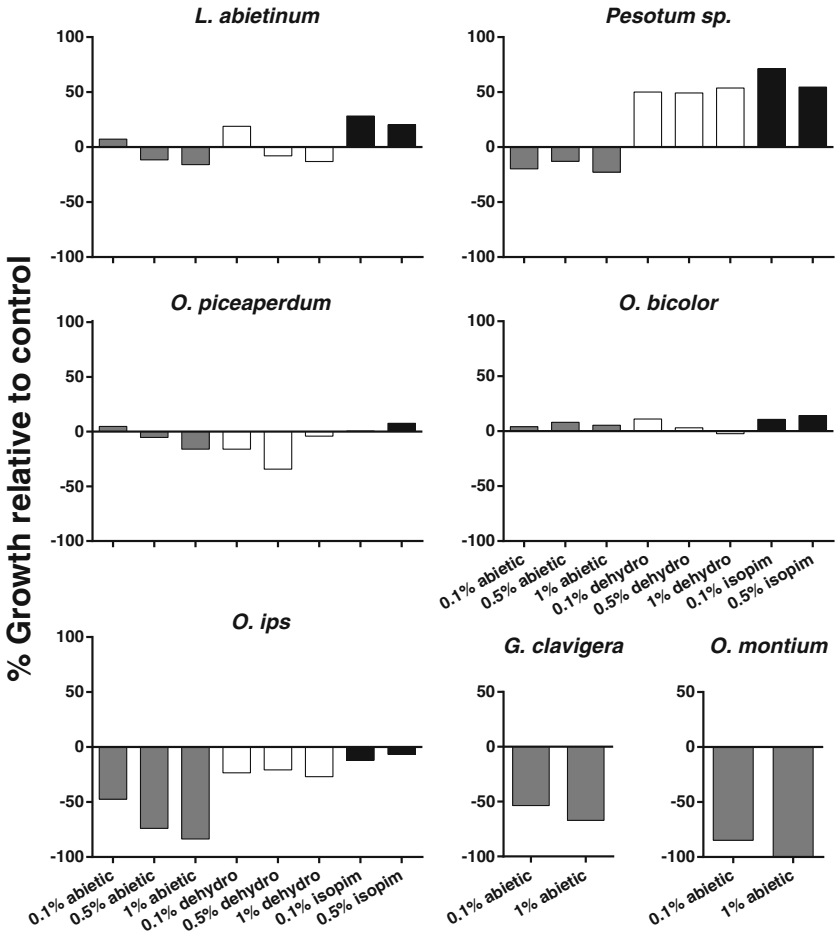

Fig 2 Responses of various bark beetle associated fungi to diterpene acids relative to the acetone control. Fungi include *Leptographium abietinum*, *Pesotum sp.*, *Ophiostoma piceaperdum*, and *O. bicolor*,the predominant symbionts of *Dendroctonus rufipennis* (3 days bioassay; this study; Table 1), *Ophiostoma ips* the predominant symbionts of *Ips pini* (3 days bioassay; Kopper et al. 2005), and *Grosmannia clavigera* and *Ophiostoma montium*, the predominant symbionts of *Dendroctonus ponderosae* (7 days bioassay; Boone et al. 2013). Negative values indicate diterpene acids inhibited growth, while positive values indicate growth stimulation. Under identical growth and experimental conditions, control treatments of *O. bicolor* and *O. ips* had comparable levels of growth (mean=11.3 and 11.25 mm, respectively)

Dehydroabietic acid stimulated growth of two of D. *rufipennis* - associated fungi, and did not affect the other two, and isoprimaric acid stimulated the growth of all fungi.

The higher tolerance of the *D. rufipennis* - associated than *I. pini* - associated fungi (Fig. 2) to diterpene acids may reflect adaptations to different host chemistries, or may explain the contrasting patterns of diterpene acid accumulation between white spruce and red pine to different treatments. That is, red pine had greater diterpene acid concentrations following fungal than mechanical challenges, which has likewise been observed in many studies of monoterpenes and phenolics in many conifer species (Boone et al. 2011; Hammerbacher et al. 2011; Klepzig et al. 1995; Villari et al. 2012; Werner and Illman 1994). The higher concentrations in spruce following mechanical than in fungal treatments is enigmatic. Since the spruce beetle-associated fungi are more tolerant of diterpene acids, it seems likely they have more active detoxification systems than fungi from other bark beetles. A second nonexclusive possibility is that bacteria associated with spruce contribute to detoxification, as bacteria that are largely or partially plant-associated have been shown to contribute to phytochemical detoxification in several systems (Adams et al. 2011; Boone et al. 2013; Mason et al. 2014; Miller et al. 2014). A third, and likewise non-exclusive possibility is that red pine primarily resists fungi using diterpene acids, whereas white spruce primarily uses other or additional chemical classes.

Accumulation of tree defense compounds to bark beetle attack is further complicated by the potential of beetle-vectored microorganisms to metabolize these compounds. Our findings with diterpene acid induction in white spruce are consistent with results describing metabolism of spruce stilbene phenolics by the fungal symbiont, *Ceratocystis polonica*, of the bark beetle *Ips typographus* (Hammerbacher et al. 2013). After initial accumulation of stilbene phenolics, *C. polonica* reduced concentrations below trees receiving only a wound inoculation. Catabolism of tree chemical defense components may be a conserved process across many bark beetle—associated fungi. Additional allelochemical metabolism may occur through additional microbes present in these systems, such as bacteria or yeasts (Boone et al. 2013; Davis and Hofstetter 2011). Currently, the extents to which these in vitro experiments scale up to whole tree colonization, and the concentrations at which these microorganisms are overwhelmed by defense compounds, are unknown.

In summary, in this study, we show that induction of diterpene acids occurs in red pine and white spruce as a component of their defense syndromes against bark beetle - fungal complexes. The responses varied between these two conifer species, in terms of total concentrations, compositional diversity, compositional changes, nature of induction, and types of treatment eliciting maximal concentration. Additionally, growth inhibition of fungal associates of the spruce beetle varied among different diterpene acids and concentrations, but overall, these fungi appear more tolerant than fungi associated with the two pine-colonizing bark beetles for which data are available. Future studies should address individual and combined effects of diterpene acids on additional bark beetle species and fungal symbionts, examine induction of diterpene acids in additional conifer species and genera, and explore mechanisms of tolerance and detoxification among multiple microorganisms associated with the beetles and their host trees.

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